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Sperm Attachment and Acrosome Reaction on the Egg Surface of the Polychaete, *Tylorrhynchus heterochaetus*[1]

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Abstract. Sperm binding to the egg envelope (chorion) was examined in fixed eggs and isolated chorions of the polychaete, *Tylorrhynchus heterochaetus*. Sperm binding included two successive steps: attachment (acrosomal outer surface-chorion binding) before the acrosome reaction and adhesion (acrosomal process-chorion binding) after the acrosome reaction. The attachment between sperm head-tip and the outermost layer of the chorion was observed in Ca-free seawater, in which the acrosome reaction did not occur. The surface of the chorion was stained with phosphotungstic acid (PTA). Sperm did not attach to pronase-treated eggs, in which the PTA-positive layer disappeared. When isolated chorions were soaked in distilled water for several hours, they lost the capacity for sperm attachment, and the PTA-positive layer thinned. The acrosome reaction was induced by material that was dissolved from the chorions into distilled water. This suggests that both the receptor for sperm attachment and the inducer of the acrosome reaction are involved in the PTA-positive layer.

Introduction

In many animals, ripe unfertilized eggs have one or more extracellular coats (envelopes). During fertilization, egg envelopes play a key role in sperm binding, in the induction of the sperm acrosome reaction, and in the exclusion of supernumerary sperm (see Epel and Vacquier, 1978; Lopo, 1983; Monroy and Rosati, 1983; Jaffe and Gould, 1985).

Previous studies on sperm-egg binding have suggested that two types of binding exist (see Epel and Vacquier, 1978): (1) binding between the outer surface of unreacted sperm heads and egg envelopes before the acrosome reaction (referred to as attachment in the present paper) in mice (Saling and Storey, 1979; Bleil and Wassarman, 1983; Wassarman *et al.*, 1985; Soldani and Rosati, 1987), ascidians (DeSantis *et al.*, 1980; Rosati, 1985), a horseshoe crab (Brown, 1976; Barnum and Brown, 1983), polychaetes (Anderson and Eckberg, 1983; Osanai, 1983; Sato and Osanai, 1983, 1986), an abalone (Lewis *et al.*, 1982) and a sea urchin (Aketa, 1973, 1975); and (2) binding between acrosomal processes of reacted sperm and egg envelopes after the acrosome reaction (referred to as adhesion in the present paper) in ascidians (DeSantis *et al.*, 1980; Rosati, 1985), a horseshoe crab (Brown, 1976), polychaetes (Osanai, 1983; Sato and Osanai, 1983, 1986), sea urchins (Summers and Hylander, 1975; Vacquier, 1980), a sand dollar (Summers and Hylander, 1974), bivalves (Hylander and Summers, 1977; Brandriff *et al.*, 1978), and a crustacean (Clark *et al.*, 1981). Studying sperm-egg binding can be difficult because, during normal fertilization in most organisms, the acrosome reaction usually follows sperm attachment too quickly to be examined. Using phase-contrast microscopy, Osanai (1983) observed that sperm remained attached to isolated egg envelopes (chorions) without the acrosome reaction in Ca-free solution, and that sperm adhered to the chorion with its acrosome reacted in Ca-containing solution in the polychaete *Tylorrhynchus heterochaetus*.

In the present study, we use electron microscopy to

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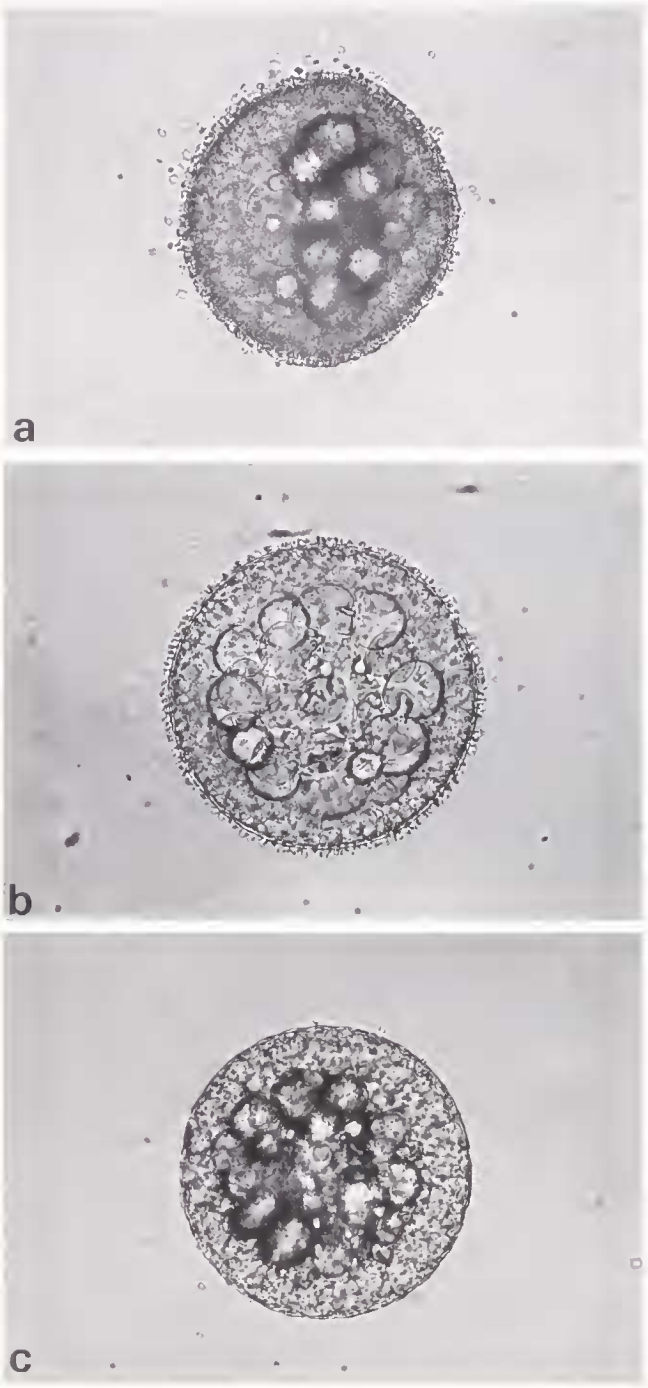

Figure 1. Sperm binding to the fixed *Tylorrhynchus* eggs. Two hours after insemination. ×260. (a) *Tylorrhynchus* sperm were bound to the egg in artificial ordinary seawater. (b) *Tylorrhynchus* sperm were bound to the egg in artificial Ca-free seawater. (c) Sperm of the sea star *Asterina pectinifera* were not bound to the egg in artificial ordinary seawater.

confirm these sperm bindings, and demonstrate that factors for the reception of initial sperm attachment and for the induction of sperm acrosome reaction are distributed in the outermost layer of the egg envelope all over the egg surface.

Materials and Methods

Preparation of gametes

Mature worms of the nereidid polychaete *Tylorrhynchus heterochaetus* were collected in Natori, Miyagi Prefecture, Japan. They were placed in 30% seawater (salinity: about 10), and refrigerated at 0–5°C. Gametes were obtained by compressing or cutting the body with forceps. Unfertilized eggs were washed several times in 30% seawater; sperm were diluted in ordinary seawater (*cf.* Osanai, 1978).

Experimental media

Natural seawater filtered with a paper filter (Toyoroshi No. 2) or Herbst's artificial seawater (ordinary and Ca-free seawater) modified by Motomura (1938) were used. Ca-free seawater was prepared by substituting NaCl for $CaCl_2$.

Isolation of chorion from eggs

Egg envelopes (chorions) were isolated from unfertilized eggs as described by Osanai (1976, 1983). The unfertilized eggs were suspended in 30% seawater and then gently homogenized with a teflon homogenizer. The homogenate was centrifuged at $300 \times g$ for 5 min. After removing the supernatant, the sedimented chorions were resuspended in fresh 30% seawater and centrifuged again. Transparent chorions were obtained by repeating this procedure several times.

Preparation of fixed eggs

The sperm-egg binding process was examined using fixed unfertilized eggs as described in sea urchins by Kato and Sugiyama (1978). The eggs were prefixed in 1% glutaraldehyde in 30% seawater for 0.5–2 h. After rinsing in 30% seawater several times, the eggs were inseminated. In other cases, unfertilized eggs were pretreated with 0.1% pronase (Kaken Chemical Co.) in 30% seawater for 20 min prior to prefixation.

Insemination

Tylorrhynchus eggs are fertilizable in media over a wide range of salinity. In this study, sperm were added to isolated chorions and fixed eggs in 30% or 100% seawater. When insemination occurred in Ca-free seawater, the chorions and eggs had been rinsed several times in Ca-free seawater, so that the concentration of contaminating Ca at insemination might be less than 1/1000 of that in ordinary seawater. Egg or chorion suspensions ($1–5 \times 10^2$/ml) were inseminated with sperm suspension (final concentration: 10^{-4} dilution of dry sperm, 5×10^6–1×10^7/ml) at room temperature (10–20°C).

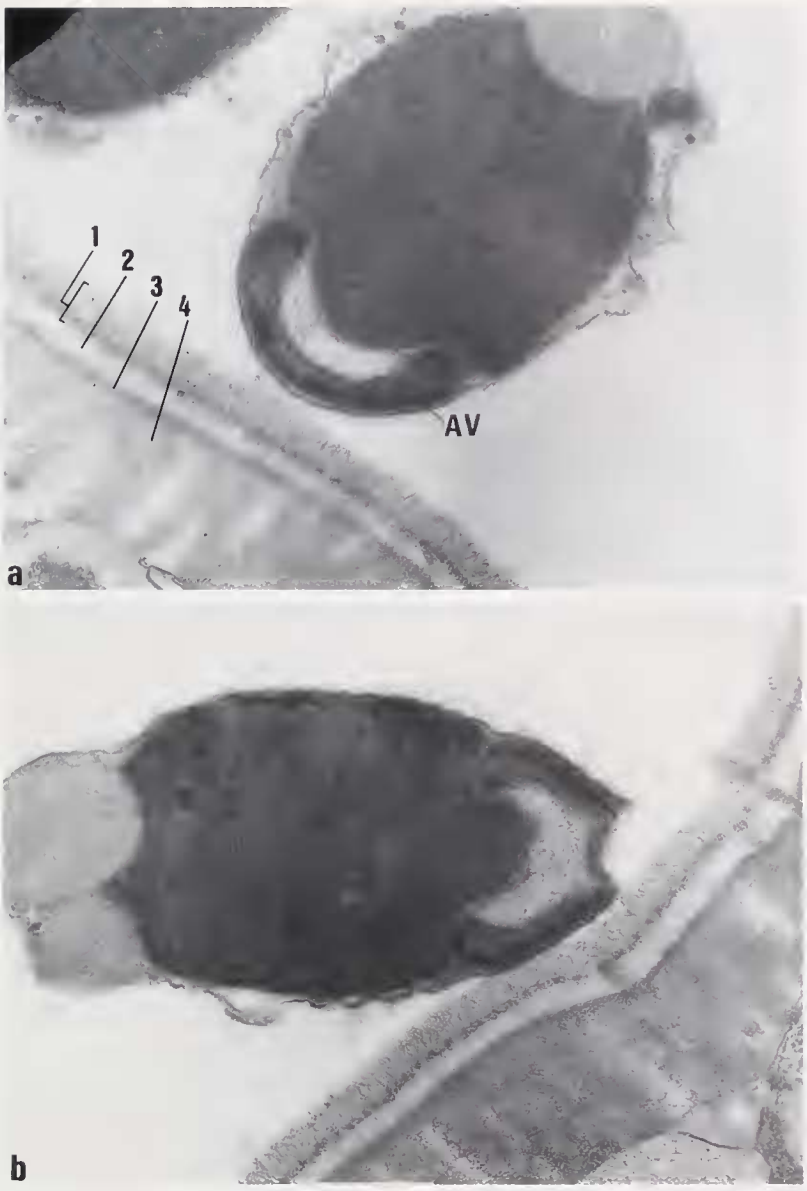

Figure 2. Attachment of unreacted sperm to the fixed eggs in artificial Ca-free seawater. One minute after insemination. ×31,500. (a) A spermatozoon attached to the first layer (1) of chorion by its head-tip without any acrosomal change. (2, 3, 4) The second, third, and fourth layer of the chorion, respectively. AV: acrosomal vesicle. (b) A spermatozoon with its acrosomal vesicle open at the head-tip. The outer membrane of sperm head was attached to the first layer of chorion.

Test of acrosome reaction-inducing activity in material dissolving from chorions

Isolated chorions were placed in distilled water (DW) (2% V/V) for 0.5–5 h. The chorion suspension was filtered through filter paper. The filtrate was diluted with ordinary seawater to 30% seawater and used as chorion extract.

Sperm suspension was added to the chorion extract (final sperm concentration: $1–5 \times 10^7$/ml). The test solution was fixed with 1–2% glutaraldehyde 15–40 min later. Sperm acrosome reaction was checked by phase-contrast microscopy (×1000).

Electron microscopy

Specimens were fixed in 2% glutaraldehyde in 70–90% seawater for several days at 0–4°C. After rinsing, they were postfixed in 1% OsO_4 in 70–90% seawater for 1 h at 0–4°C. The specimens were dehydrated in ethanol and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate or with 10% phosphotung-

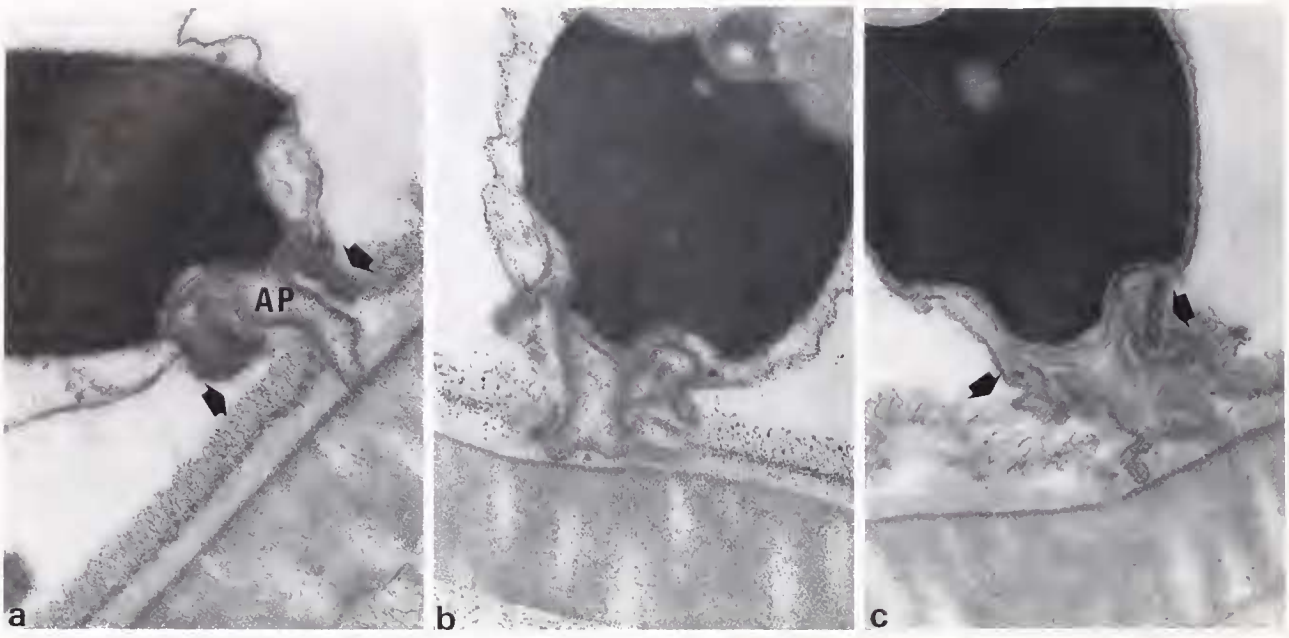

Figure 3. Adhesion of reacted sperm to the fixed eggs in artificial ordinary seawater (a, b, c). One minute after insemination. The acrosomal process (AP) penetrated the first and second layers of chorion and adhered to the third layer. The outer membrane of acrosome was in contact with the fibrous component of the first layer of chorion (arrows). ×35,000.

stic acid (PTA), and then examined with a transmission electron microscope.

Results

Sperm binding to fixed eggs

In both ordinary seawater and Ca-free seawater, *Tylorrhynchus* sperm were bound to the surface of fixed eggs at their head-tip (Fig. 1a, b). When the fixed eggs were inseminated with sperm of the sea star *Asterina pectinifera* and the sea urchin *Strongylocentrotus nudus*, the sperm were not bound to the eggs (Fig. 1c).

The chorion (1–1.5 μm thick) consists of four layers (Fig. 2; see also Sato and Osanai, 1983). The first (outermost) layer is composed of a row of small packed spheres wrapped in fibrous matter. The second layer is composed of less electron-dense material. The third layer is a thin electron-dense layer. The fourth layer (innermost and thickest) is composed of densely packed material with many cavities opening toward the inner surface.

The ultrastructure of the sperm-egg binding was examined with specimens fixed 1 min after insemination. In Ca-free seawater, most sperm were attached to the first layer of chorion by their head-tip without any acrosomal change, though the acrosomal vesicle had opened in a few sperm (Fig. 2). The opening of the acrosomal vesicle was sometimes observed in free sperm suspended in seawater, and the usual morphological change associated with a true acrosome reaction did not occur. Thus, opening of the vesicle may be either a spontaneous phenomenon or an artifact of fixation. In any case, the outer surface of the sperm head-tip made contact with the fibrous component of the first layer.

When live eggs were inseminated in Ca-free seawater, sperm temporarily attached to the egg surface, but soon detached. The sperm-egg attachment without a sperm acrosome reaction seems to be less stable in live eggs than in fixed eggs.

In ordinary seawater, most sperm bound to the chorion underwent the acrosome reaction and formed a lobular acrosomal process (Fig. 3). The acrosomal process penetrated the first and second layers of the chorion and adhered to the third layer. At the same time, the outer membrane of the acrosome (the lateral side of the opened acrosomal vesicle) continued to contact the fibrous component of the first layer of the chorion. Some sperm that had not undergone the acrosome reaction were attached to the first layer of the chorion.

Sperm binding to isolated chorions

In intact unfertilized eggs, the first layer of the chorion was stained by PTA (Fig. 4a, see also, Sato and Osanai, 1983). The outer surface of the isolated chorion (morphologically similar to the third layer) was stained with PTA (Fig. 4b), suggesting that the first and second layers had been deformed or had collapsed onto the third layer.

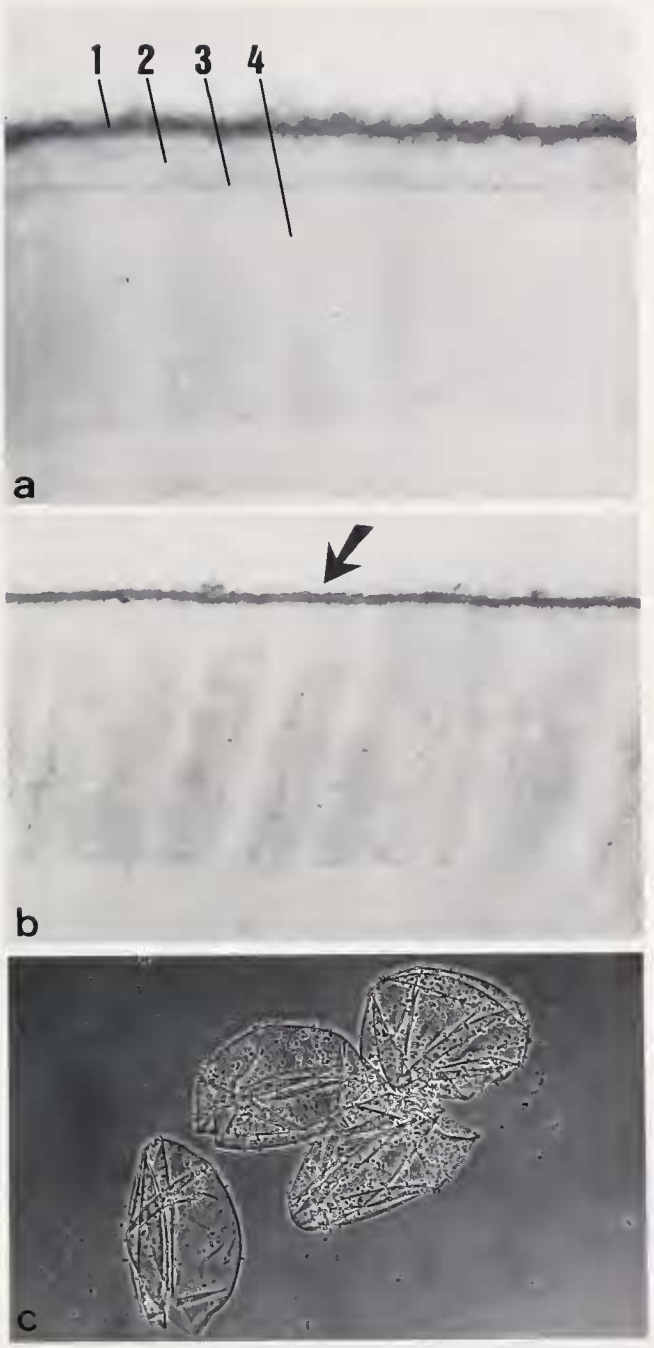

Figure 4. (a) Ultrastructure of the surface of an intact unfertilized egg. A section stained with phosphotungustic acid (PTA). The first layer (1) of the chorion was stained intensively. (2, 3, 4) The second, third, and fourth layer of the chorion, respectively. ×40,800. (b) Ultrastructure of the isolated chorion. A section stained with PTA. The outer surface (arrow), which was morphologically similar to the third layer, was stained. ×45,600. (c) Sperm binding to the isolated chorions in artificial ordinary seawater. Two minutes after insemination. ×140.

Sperm were bound to the isolated chorion in both ordinary and Ca-free seawater, and the bindings of sperm were kept for a long time (Fig. 4c). Because the isolated chorion was transparent, the acrosome reaction of the attached sperm could be checked by phase-contrast microscopy. In ordinary seawater, many sperm underwent the acrosome reaction (Table I, Fig. 5). These sperm were also examined by electron microscopy. The lobular acrosomal process adhered to the outer surface of the chorion and did not penetrate it (Fig. 6a). In Ca-free seawater, the

Table I

Percentage of occurrence of acrosome reaction in sperm bound to isolated chorion in presence or absence of Ca^{2+}

Expt. No.	Percentage of acrosome reaction	
	Ordinary seawater	Ca-free seawater
1	83.0	0.4
2	64.4	0
3	61.3	0.2
4	11.6	0

Isolated chorions were fixed 1–10 min after insemination. Occurrence of acrosome reaction was checked by phase contrast microscopy in 200–300 spermatozoa on 3–6 chorions.

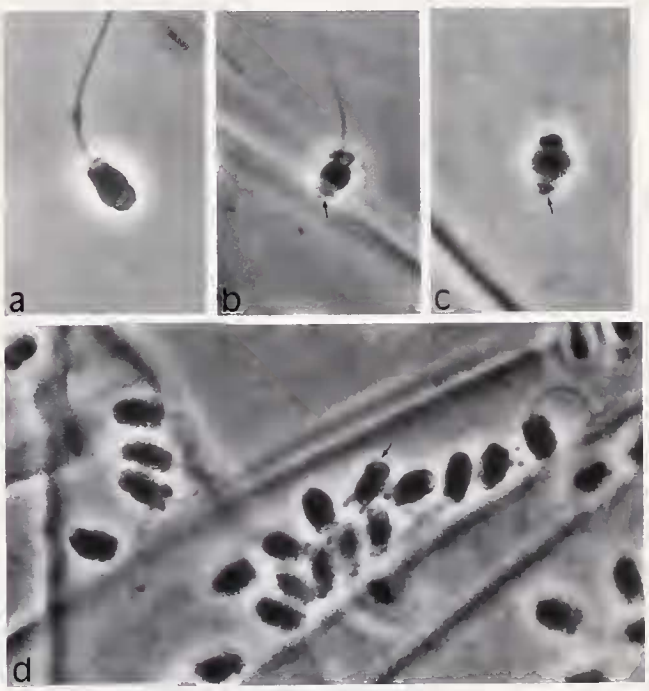

Figure 5. Phase contrast micrographs of sperm binding to the transparent isolated chorions. ×1900. (a) Control. An intact free-swimming spermatozoon. (b, c) Spermatozoa undergoing acrosome reaction and adhering to the chorion in artificial ordinary seawater. Ten minutes after insemination. Arrows indicate the development of acrosomal process. (d) Spermatozoa attached to the chorion without acrosome reaction in artificial Ca-free seawater. An arrow indicates an intact acrosomal vesicle. Ten minutes after insemination.

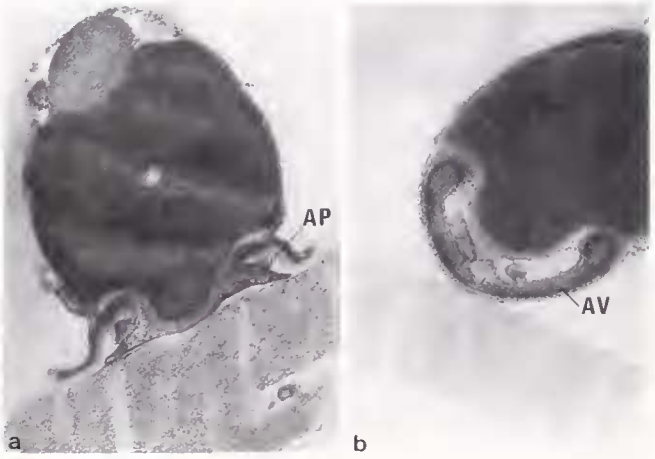

Figure 6. Electron micrographs of sperm binding to the isolated chorion. One minute after insemination. ×24,800. (a) A spermatozoon adhering to the outer surface of chorion with the spread acrosomal process (AP) in artificial ordinary seawater. (b) A spermatozoon attached to the outer surface of the chorion without acrosome reaction in artificial Ca-free seawater. AV: Acrosomal vesicle.

sperm bound to the isolated chorion did not undergo the acrosome reaction (Table I, Fig. 6b). The outer acrosomal membrane of sperm head-tip was attached to the chorion surface. No spermatozoon was bound to the inner surface of the chorion (the fourth layer) in both ordinary and Ca-free seawater.

Effect of pronase treatment of eggs on sperm-egg binding

We tried to remove the egg-surface component that binds sperm and induces the acrosome reaction. Unfertilized eggs were pretreated with pronase and then fixed. After rinsing, the eggs were inseminated in 30% seawater. Sperm attachment was blocked or greatly reduced (Table II, Fig. 7).

The pronase-treated eggs were examined by electron microscopy. The first and the second layers were removed from the chorion in the pronase-treated eggs (Fig. 8). The outer surface of the chorion did not stain with PTA.

Acrosome reaction-inducing activity in chorion extract

Chorion extracts were prepared by soaking chorions in DW. Sperm were added to the chorion extract diluted with natural seawater. Many of the free swimming sperm underwent acrosome reaction (Fig. 9, Table III). The sperm did not undergo acrosome reactions in control media (30% seawater).

After isolated chorions were treated with DW, they were mixed with sperm in 30% seawater. Few sperm were bound to the chorions (Fig. 10). These chorions were examined by electron microscopy. The PTA-positive layer at the outer surface of the chorions became thinner after the DW-treatment (Fig. 11). No morphological change was observed in other parts of chorion.

Discussion

Osanai (1983) used light microscopy to examine sperm binding to the isolated chorion in *Tylorrhynchus heterochaetus*. He showed that sperm binding includes two steps: sperm attachment before the acrosome reaction and sperm adhesion after the acrosome reaction. He also showed that the progression from sperm attachment to adhesion requires external calcium ions. We used electron and light microscopy to observe sperm-chorion binding using fixed eggs and isolated chorions. Our results confirm the validity of Osanai's (1983) report.

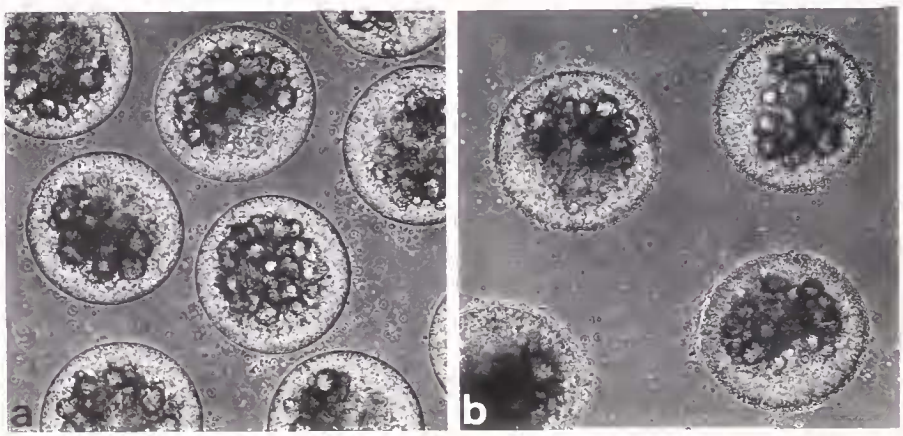

Figure 7. Inhibition of sperm binding by pronase-pretreatment of eggs. The eggs were inseminated in 30% natural seawater after glutaraldehyde-fixation, and observed 10 min after insemination. ×140. (a) The eggs pretreated with pronase for 20 min. Sperm did not bind to the eggs. (b) The eggs without pronase-pretreatment. Many sperm bound to the eggs.

Table II

Decrease of sperm binding by pronase-pretreatment of eggs

Eggs	No. of sperm bound on the egg contour*
Pronase-treated	3.2 ± 0.7 (n = 20)
Untreated	66.5 ± 3.6 (n = 12)

* Average ± SD (No. of eggs examined).

Sperm attachment and adhesion were demonstrated ultrastructually in Ca-free seawater and ordinary seawater, respectively. Both were also photographed 1 min after insemination during normal fertilization (Sato and Osanai, 1983). However, in normal fertilization, the sperm attachment step is rather inseparable, because it is followed quickly by the acrosome reaction. We could separate the sperm attachment step in Ca-free medium, in which the acrosome reaction was prevented.

Sperm attachment occurred at only the head-tip of conspecific sperm. This appears to be a species-specific and site-specific reaction for the first sperm-egg recognition. Specific attachment between sperm and egg envelope before the acrosome reaction is also known in an ascidian (Rosati and De Santis, 1978) and in mammals (Wassarman *et al.*, 1985). Sperm attachment was inde-

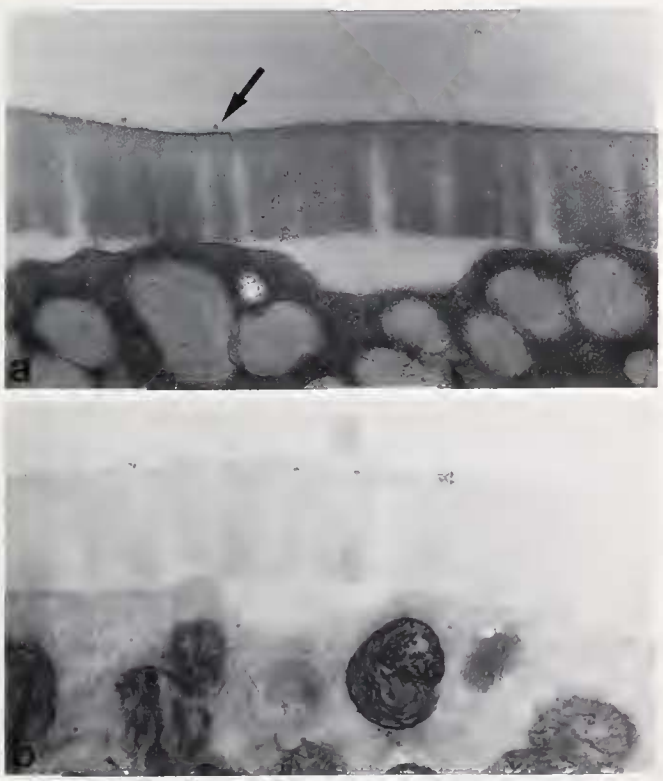

Figure 8. Ultrastructure of the pronase-treated eggs. The eggs were treated with pronase for 20 min. ×24,000. (a) A section stained with uranyl acetate and lead citrate. Most parts of the first and second layers of the chorion disappeared with a few spherical components of the first layer remaining on the surface (arrow). (b) A section stained with phosphotungstic acid. The outer surface of the chorion was not stained as compared with the untreated egg and the isolated chorion (Fig. 4).

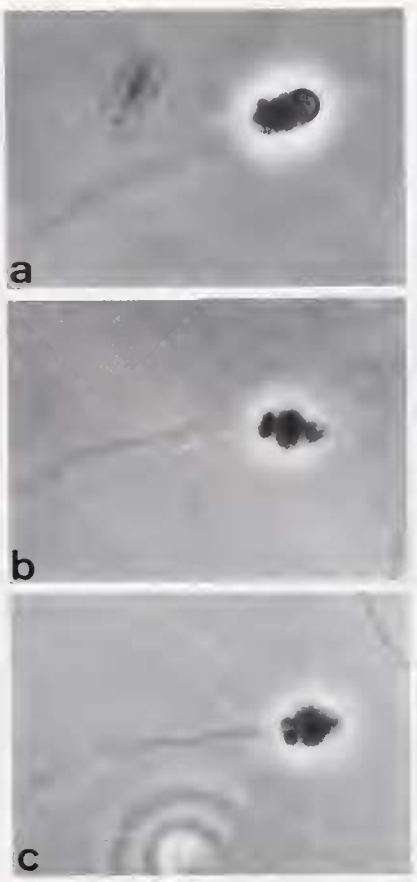

Figure 9. Induction of acrosome reaction by the chorion extract. ×2,100. (a) Unreacted sperm in control medium (30% seawater). (b, c) Sperm undergoing acrosome reaction in the chorion extract.

Table III

Acrosome reaction-inducing activity of the chorion extract

Expt. No.	Duration of incubation in distilled water (h)	Percentage of acrosome reaction*	
		Extract	Control**
1	0.5	82.4	0
2	1.5	3.7	2.7
3	5	77.4	1.9
4	5	19.1	7.6

* Occurrence of acrosome reaction was checked in 100–150 spermatozoa by phase contrast microscopy.

** 30% natural seawater.

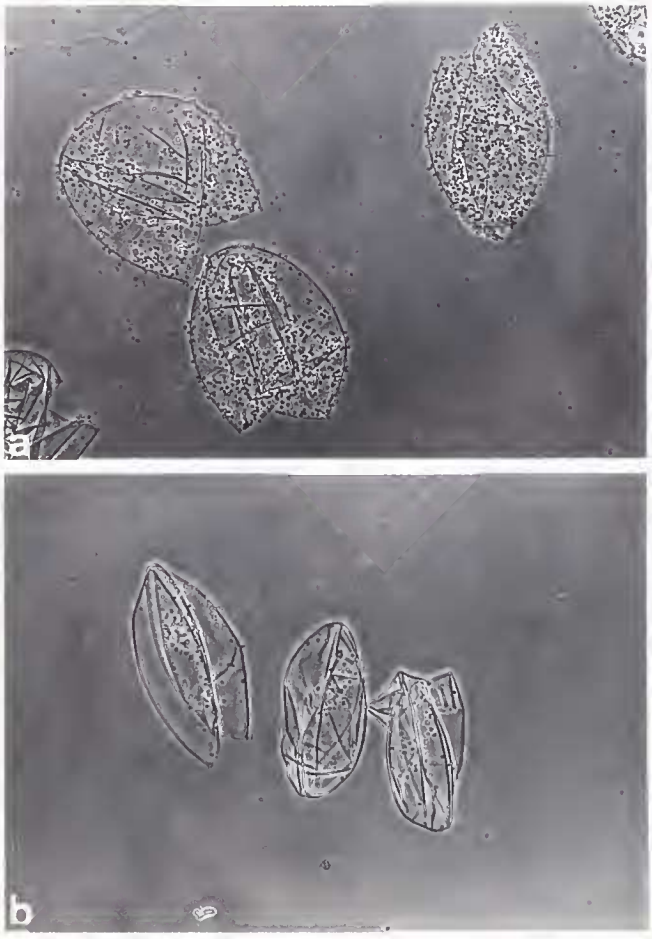

Figure 10. Decrease of sperm binding by soaking of the isolated chorions in distilled water. The isolated chorions were inseminated in 30% natural seawater just after preparation (a) or after distilled water-treatment of the chorions for 90 min (3 times). Many sperm bound to the chorion in the former (a), but not in the latter (b) 30 min after insemination. × 130.

pendent of external calcium ions in *Tylorrhynchus heterochaetus*, while it was calcium-dependent in mouse eggs (Saling *et al.*, 1978; Saling and Storey, 1979; Soldani and Rosati, 1987). Why sperm attachment to live *Tylorrhynchus* eggs is less stable than to fixed eggs or isolated chorions, in Ca-free seawater, is unknown. The outermost layer of the chorion of live eggs may be less tightly fastened to the chorion proper. Alternatively, attached sperm may be detached by a factor secreted from live eggs, as in normally fertilized eggs (Osanai, 1976). However, it is unknown whether unfertilized eggs secrete the sperm-detaching factor in Ca-free solution.

The acrosome reaction in *Tylorrhynchus heterochaetus* evidently requires external calcium ions as in sea urchins (Dan, 1954; Collins and Epel, 1977). The jelly, the outermost layer of the sea urchin egg envelope, induces the acrosome reaction and sperm aggregation behavior (see Epel, 1978). Sperm aggregation can be induced even in a medium of low Ca^{2+} concentration, in which the acrosome reaction is prevented (Dan, 1954). Aggregation-inducing and acrosome reaction-inducing factors were separated from jelly of a starfish (Uno and Hoshi, 1978). Uno and Hoshi (1978) considered that sperm aggregation might reflect the initial sperm-egg interaction, *i.e.*, the sperm attachment to the jelly surface.

In intact *Tylorrhynchus* eggs, the PTA-positive fibrous substance coats the outer surface of chorion. Our results show that both the receptor for sperm attachment and the inducer for the acrosome reaction are associated with the PTA-positive layer. It is unknown whether the components of the PTA-positive layer function differently or whether a single factor has both functions. Because the PTA-positive layer corresponds to a periodic acid-Schiff (PAS)-positive one observed by light microscopy (Sato and Osanai, 1983) and is digested by pronase, it seems to contain polysaccharide and protein. The glycoprotein ZP3 of mouse zona pellucida is responsible for both sperm attachment and the induction of the acrosome reaction (Bleil and Wassarman, 1983). Other reports

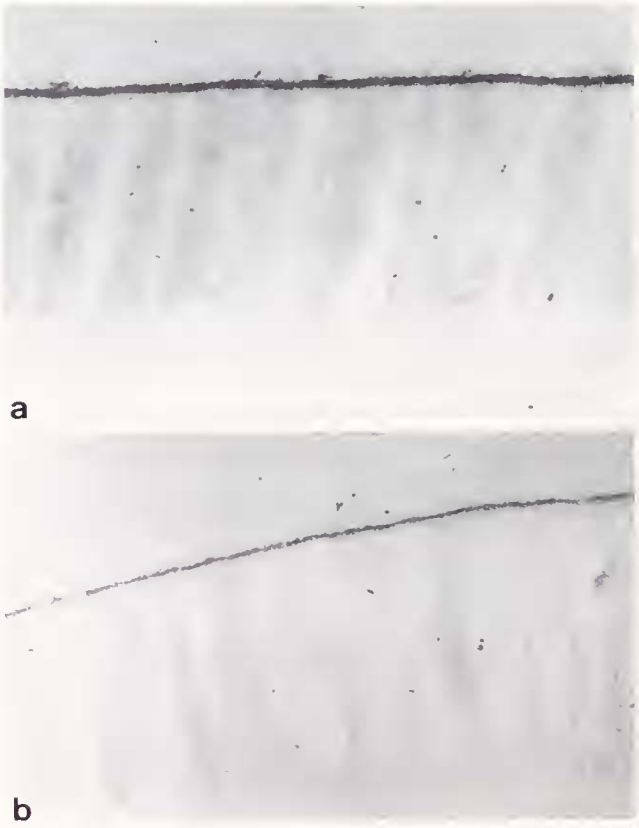

Figure 11. Ultrastructural alteration of the isolated chorion by the distilled-water treatment. Sections were stained with phosphotungstic acid (PTA). × 36,700. (a) An isolated chorion just after preparation. (b) An isolated chorion after distilled-water treatment for 60 min. The PTA-positive layer at the outer surface of the chorion became thinner.

showed that a sugar or glycoprotein on an egg envelope plays an important role in sperm attachment or induction of the acrosome reaction in the eggs of a mouse (Shur and Hall, 1982a, b), an ascidian (Rosati and De-Santis, 1980; Pinto *et al.*, 1981), a horseshoe crab (Barnum and Brown, 1983), a sea urchin (Segall and Lennarz, 1979; Yoshida and Aketa, 1983), a seastar (Uno and Hoshi, 1978), and a bivalve (Tumboh-Oeri and Koide, 1982).

The acrosomal process of the fertilizing spermatozoon fuses with a microvillus projecting from the egg through the chorion in *T. heterochaetus* (Sato and Osanai, 1983). However, no morphological difference of the PTA-positive layer was observed between regions around microvilli and the other regions. In contrast with *T. heterochaetus*, the acrosome reaction-inducing activity was localized to a limited number of specialized sites above egg microvilli in the nereidid polychaete *Neanthes japonica* (Sato and Osanai, 1986).

Sperm seem to initiate the acrosome reaction just after attaching to the chorion in the presence of Ca. Their acrosomal processes usually penetrate the first and second layers of the chorion and adhere to the third layer. Such sperm can fuse with an egg microvillus to fertilize the egg (Sato and Osanai, 1983). The initial attachment between the sperm head-tip and the outermost layer of chorion may be important for keeping the sperm oriented for successful fertilization during the acrosome reaction. Holding the sperm erect on the chorion surface should lead to proper penetration and adhesion of the acrosomal process.

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